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Award Number: DAMD17-00-1-0222

TITLE: Survival Pathways and Apoptosis of Breast Cancer Cells as Targets for Therapeutic Modulation

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REPORT DATE: May 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2001	Annual (1 May 00 - 30 Apr 01)

4. TITLE AND SUBTITLE
Survival Pathways and Apoptosis of Breast Cancer Cells as Targets for Therapeutic Modulation

5. FUNDING NUMBERS
DAMD17-00-1-0222

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
See Report

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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RESEARCH PROGRESS

In the past year I have assessed the dependence of breast cancer cell survival on the PI3 kinase/Akt pathway. The ability of cells to survive under adverse conditions, such as when growth factors are limited or when chemotherapeutics are present, likely contributes to the progression of breast cancer. Using six breast cancer cell lines and one immortal, but phenotypically normal, breast line, I first looked at the ability of each line to survive under serum-free conditions. Next I assayed the effects of two pharmacological inhibitors of key survival signaling pathways on breast cancer cell survival both under serum-free and serum-containing conditions. The results suggest that most breast cancer cell lines rely more heavily on the MEK1/2→ERK1/2 signaling pathway than on the PI3-kinase→Akt pathway for survival.

Survival was measured by quantitating the number of viable cells in a culture over time. Viability was determined by exclusion of trypan blue dye. I also looked at the effect of serum-free media on the proliferative potential of breast cancer cells. Important survival signals originate from outside the cell. Growth factors, through interactions with their receptors, have been found to modulate both growth and survival signaling pathways. By eliminating serum, I removed all exogenous growth factors from the extracellular environment. It was expected that serum removal would limit growth. To test whether the viable cells, which remained in each culture after various days of serum-free media, retained the ability to proliferate, I developed a simple assay to measure proliferative potential. Following exposure to adverse conditions, cells were re-plated in serum-containing media. Growth was assessed by measuring the increase in DNA content per well over a series of days. This type of assay was also used to determine the effects of various chemotherapeutics on proliferative potential. In addition to providing information about the cytotoxic or cytostatic effect of a given treatment, the method of measuring viability and proliferative potential provides critical information with regard to the ability of the remaining cells to repopulate under less-stressful conditions. Together these measurements may more accurately model what occurs *in vivo* following treatment.

Survival time under serum-free conditions varies among the cell lines. Several of the breast cancer cell lines, as well as the phenotypically normal MCF-10A line, contained a significant portion (>40% of the number initially plated) of viable cells after more than 20 days in serum-free media. This suggests that the absence of exogenous growth factors does not induce an immediate death in these lines. It is possible that these cells have altered signaling pathways which obviate the need for growth factors or that they secrete enough growth factors to keep the population alive. Two of the breast cancer cell lines, MDA-MB-468 and ZR-75-1, actually grew significantly in serum-free media. Two other lines, SKBr3 and T47D, double once and then began to lose viability rapidly. The remaining lines did not appear to grow in serum-free media and essentially kept a constant number of viable cells over time. In most cases, the viable cells retained proliferative potential. I also found that survival time in suspension, which limits survival signals emanating from the cell matrix, varied among the cell lines.

Viability and proliferative potential of all seven cell lines correlated with the presence of phospho-ERK1/2 expression. In the ZR-75-1 cell line, viability and proliferative potential correlated with the presence of both phospho-ERK1/2 and phospho-Akt. While phospho-Akt was present in some of the other cultures that survived, there was no case in which only phospho-Akt in the absence of phospho-ERK correlated with survival. These data suggest phospho-ERK, rather than phospho-Akt, correlates with breast cancer survival under serum-free conditions.

I next tested the effect of pharmacologic inhibitors of MEK1/2 and PI3-kinase signaling on breast cancer cell survival. U0126 and PD98059 were used to inhibit MEK1/2. LY294002 was used to inhibit PI3-kinase signaling. Since these signaling pathways are linked to both growth and survival, I measured the effect of the inhibitors on proliferation, cell-cycle status, viability and proliferative potential. The phosphorylation status of downstream targets (mainly phospho-ERK1/2 and phospho-Akt) was also measured in order to determine the effectiveness of the inhibitor on signaling. These studies were conducted in both serum-free and serum-containing conditions.

U0126 or LY294002 inhibited the proliferation of all seven breast cell lines. Preliminary cell cycle data show that these agents induce a G1/S-phase block. U0126 treatment actually resulted in a complete loss of viability and proliferative potential over time. However, there was a differential in response time between the cell lines. MDA-MB-231 and T47D cells were all dead after 4 or 5 days of U0126 treatment, respectively. All of the breast cancer cell lines contained 100% non-viable cells after 7 days of continuous U0126 exposure under serum-free conditions. Notably, the MCF-10A cells, while growth inhibited, maintained a viable subpopulation with proliferative potential over this period. The difference between cell lines in survival time under serum-containing *vs.* serum-free conditions plus U0126 was negligible. An exception to this finding was the MDA-MB-468 and MCF-10A lines, which survived for several more days when serum was present. Unlike U0126, LY294002 treatment was not found to result in total cell death except for in the ZR-75-1 cell line. As noted earlier, survival in this cell line correlated with the presence of both phospo-ERK1/2 and phospho-Akt. The ability of U0126 and LY294002 to eliminate survival suggests that in ZR-75-1 cells, both the MEK1/2→ERK1/2 and PI3-kinase→signaling pathways are required for survival.

The results described above have completed the aims of Task 1 and are being prepared for publication. Due to these findings, the aims of Task 2 will be modified to put more emphasis on the effectors of the MEK1/2→ERK1/2 signaling pathway. I will also be looking at the importance of effectors common to both pathways, such as phospho-Bad. Cells treated with U0126 can be rescued if the compound is removed early enough. However after a certain critical point, cells can no longer be rescued. I am currently looking at cell cycle and other markers to determine what changes in protein expression occur at this critical point.

The aims of Task 3 are starting to be studied. Some experiments have been conducted to test the efficacy of combining U0126 or LY294002 with currently used

chemotherapeutics. Using the cell culture model, I have begun to test the effects of combining adriamycin, taxol, 5-fluoruracil, navelbine or herceptin with U0126 and/or LY294002. Some of the cell lines do not display the hallmarks of apoptosis. For example, MDA-MB-468, SKBr3 and ZR-75-1 cells display DNA ladders when they are dying and the other lines do not. For this reason, I will focus on the effects of the drug combinations on viability, proliferative potential and protein profiles.

TRAINING

Over the past year I have presented my research at bi-weekly lab group meetings, attended weekly seminars in the Department of Pharmacology and Toxicology, monthly seminars in the Norris Cotton Cancer Center, and monthly Molecular Therapeutics meetings. I will be presenting my findings at the AACR annual meeting in March 2001. Currently, manuscripts are being prepared to describe these findings.

KEY RESEARCH ACCOMPLISHMENTS

Task 1: Assess the dependence of breast cancer cell survival on PI3 kinase/Akt pathway

- Survival time of breast lines in serum-depleted media is varied.
- Survival time of breast lines in suspension is varied.
- The MEK1/2 inhibitor, U0126, reduces survival time in both serum-containing and serum-depleted media in all (6) of the breast cancer cell lines tested.
- The PI3 kinase inhibitor, LY294002, has little to no affect on survival time of 5 of the breast cancer cell lines in serum-containing and serum-depleted media.
- LY294002, reduces survival time of the ZR-75 breast cancer cell line in serum-containing and serum-depleted media.
- Under the above conditions, cells that maintain a viable subpopulation often, but not always, retain proliferative potential.
- Under serum-free and serum-containing conditions LY294002 arrests breast cancer cell growth, but does not eliminate proliferative potential (exception: ZR-75-1 cells).

Task 2: Define downstream effectors of PI3 kinase/Akt pathway

- Under serum-free conditions, all cultures that retained a viable sub-population and proliferative potential expressed phospho-ERK1/2.
- Under serum-free conditions, some of the cultures that retained a viable sub-population and proliferative potential expressed phospho-Akt.
- Viability and proliferative potential of ZR-75-1 cells correlated with the presence of both phospho-ERK1/2 and phospho-Akt.
- In no case did viability and proliferative potential correlate with the presence of phospho-Akt alone.

Task 3: Determine how selected anticancer agents deregulate survival pathways to induce apoptosis

- Survival curves of each line exposed to adriamycin, taxol, 5-fluorouracil and navelbine $\pm$ U0126 or LY294002.

EXTENDED ABSTRACT*

The ability to maintain viability under stressful conditions is a characteristic of many cancer cells that may be important in carcinogenesis and may determine the effectiveness of cancer treatment. We are studying the role of two key signaling cascades, phosphatidylinositol 3-kinase (PI3K)→Akt and MEK→extracellular signal-regulated kinase (ERK), in the survival of seven different breast cell lines (MCF-10A, an immortalized, phenotypically normal line and 6 cancer cell lines). When cultured in serum-free media to eliminate exogenous survival factors, the cell lines died at markedly different rates. For example, >95% of SKBr3 cells died within 8 days, while in MDA-MB-468 and ZR75.1 cultures, the number of viable cells present after 14d in serum-free media was greater than the number initially plated. To determine whether cells retain the ability to proliferate after a stressful insult, we developed a simple model that measures both viability and proliferative potential. Following an insult, regrowth was assessed in serum-containing media using a 96-well format. This experimental model may be relevant to the *in vivo* situation in which treatment of tumors causes death in only a fraction of the population leaving some cells with the ability to regrow. In serum-free media, some cell cultures retained cells with proliferative potential for <14 days (MCF-7, SKBr3), while other lines contained cells that regrew after >50 days (MDA-MB-468, ZR-75.1). In the breast cancer cell lines, regrowth correlated with the presence of phospho-ERK. In the ZR75.1 line, regrowth correlated with the presence of both phospho-Akt and phospho-ERK. In no case did proliferative potential correlate with the presence of phospho-Akt alone. These results suggest that the MEK→ERK pathway is necessary for breast cancer cell survival and regrowth. Treatment with the MEK inhibitor, U0126 (20 μM), dramatically reduced the survival of all six breast cancer cell lines. Even under serum-containing conditions, 100% of the cells were dead in <2-6 days except for MDA-MB-468, which died after 15 days. In contrast, the MCF-10A line retained viable cells with proliferative potential after >15d of U0126 treatment. Treatment with the PI3K inhibitor, LY294002 (20 μM), for 6 days arrested or slowed the growth of all of the cell lines but unlike U0126, did not cause death. These results suggest that the ERK pathway may be more critical for survival than the PI3K pathway in these breast cancer cell lines and may represent a valid therapeutic target.

*manuscript in preparation